INVESTIGATION OF KERATINOCYTE DIFFERENTIATION AND PROLIFERATION MARKERS

PhD Dissertation

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1. INTRODUCTION

The epidermis, the outermost skin layer, provides the first line of defense against the external environment. The major cell type in epidermis and the cell type responsible for constructing the protective barrier is the epidermal keratinocyte [1,2,3]. Keratinocyte differentiation is the process whereby a relatively undifferentiated keratinocyte is converted into a suprabasal transiently amplifying cell (TAC) and finally into a corneocyte. The different populations of differentiating epidermal keratinocytes are characterized by expression of specific markers and they are functionally distinct. The transition from a basal cell to a corneocyte is a complex process that requires the simultaneous activation and inactivation of a wide variety of genes. Although it is unclear what mechanisms regulate the exit from cell cycle into senescence in keratinocytes, both the functional down-regulation of integrins and the expression of certain types of keratins (e.g. keratins 1 and 10 (K1/K10)) seem to be involved in this fundamental step in the differentiation program [4,5,6]. A major goal of keratinocyte-related research is to identify the mechanisms that regulate these processes.

1.1 Proliferation and differentiation markers in the epidermis

While most stages of squamous differentiation are well characterized, including changes in gene expression, factors that regulate the induction and repression of genes remain largely unknown. Integrins and keratins are two major groups of proteins that are closely related to keratinocyte proliferation and differentiation [4,6,7,8,9,10]. Basal keratinocytes contain several integrins, the proteins that recognize extracellular matrix components. Integrins are transembrane proteins composed of one α- and one β-subunit. The particular αβ combination determines the specificity of binding of an integrin to its extracellular matrix protein ligand. Keratinocyte integrins $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_4$ and $\alpha_v\beta_5$, are receptors for collagen, laminin, fibronectin, kalinin and vitronectin, respectively.

α5 integrin is poorly expressed in normal skin but in highly proliferative skin such as foetal epidermis, wounded skin and in psoriasis its expression is up-regulated [11,12,13,14]. In all cases, α5 integrin is expressed only in undifferentiated keratinocytes and it is lost from

the cell surface as keratinocytes differentiate [4,5,10]. Recent evidences suggest that integrins function not only as adhesive proteins but also as receptors capable of transducing biochemical signals to the interior of the cell [15]. Fibronectin through its main receptor, $\alpha5\beta1$ integrin, can provide anchorage-independent direct cell cycle regulatory signals [16] by the direct modulation of cyclin-dependent kinase activity [17]. Chen et al. showed that integrin mediated cell adhesion activates mitogen activated protein kinases (MAP kinases) [18]. In *ex vivo* keratinocyte culture anti-$\alpha5$ integrin monoclonal antibody inhibits the growth of already adhered keratinocytes on fibronectin [19].

The commonly used phenotypic markers of epithelial development and differentiation are keratins, a large family of approximately 30 proteins that form the intermediate filament network in all epithelial cells [20]. The most prominent proteins specific to basal cells are keratins 5 and 14. Keratins 1 and 10 are expressed in differentiating keratinocytes, while keratins 6 and 16 are expressed in activated keratinocytes [21]. Keratin genes are not dispersed through the genome but clustered in linkage groups [22].

For a long time it was thought that K1/K10 keratins have only structural role in the cells. More recent data suggest that in addition to providing mechanical integrity to the cells in the context of a tissue, keratins also participate in signaling processes fundamental for cell physiology [23]. Ectopic expression of K10 has been shown to inhibit the proliferation of HaCaT keratinocytes [23], although in the *in vivo* epidermis keratinocytes in the immediate suprabasal compartment (that already express K1/K10) can still proliferate ($K1/K10^+$ transiently amplifying keratinocytes) [24].

Progression through G1 phase to the DNA synthesis (S) phase of the mammalian cell cycle is controlled by D-type (D1, D2 and D3) cyclins in conjunction with their catalytic partners, cyclin-dependent kinase 4 (CDK4) and CDK6 [25]. One well-appreciated role of cyclin D-dependent kinases is to trigger the phosphorylation of the retinoblastoma protein, thereby helping to cancel its G1-specific growth-suppressive function [26,27]. Cyclin D is identified as the point at which growth factors act on cell cycle regulation [25]. In their monomeric states, CDK subunits are inactive and their catalytic activity depends on cyclin binding [28]. Abnormalities in cell cycle regulating mechanism such as increased activity of cyclin-CDK due to mutation of p53 protein and overexpression of cyclin D in both human

squamous cell carcinoma of the skin and experimentally produced SCC originating from mouse keratinocytes (KC) have been reported [29-33]. Forced expression of cyclin D1 in epithelial tissues of transgenic mice results in epidermal hyperproliferation. This fact suggest that cyclin D1 can modulate epithelial proliferation [34]. Miracco et al. showed that the expression of cyclin D1 decreases after cyclosporin treatment of the hyperproliferative skin disease, psoriasis [35]. In mammals three types of D-types cyclins exist that are named cyclin D1, D2 and D3 that share an average of 57% identity over the entire coding region and 78% in the cyclin box. Since growth factor regulated expression of the D-type cyclins varies in different cell types [25], D-type cyclins may play different functional roles in various cell types by responding to different signal transduction pathways. In mitogenically stimulated peripherial T cells, cyclins D2 and D3, but not D1 are induced during G1 transition. In contrast with this, in macrophages stimulated to enter the cell cycle with colony-stimulating factor 1, cyclins D1 and D2 but not D3 are produced during G1 phase. Thus different D-type cyclins are likely to be not redundant, but to play distinct roles in mammalian cells. Although both D1 and D2 cyclins are expressed in normal human keratinocytes and in the non-tumorigenic HaCaT keratinocyte cell line, the specific roles for D1 and D2 type cyclins in keratinocytes remain unknown.

HaCaT cells, although immortalized and genetically abnormal, are considered to be good models for human keratinocytes [36,37], therefore they are commonly used in experiments examining effects of therapeutic drugs on keratinocyte physiology and studying keratinocyte biology [23,38,39,40]. HaCaT cells form an almost normal epidermis with similar architecture to that formed by normal adult keratinocytes, including the formation of basement membrane and keratinization, when transplanted on nude mice or cultured in organotypic culture with human dermal fibroblasts [41,42]. However, the formation of normal tissue architecture is delayed in HaCaT transplants, indicating a reduced sensitivity to environmental signals compared to adult epidermal keratinocytes [41].

It is known that similar to normal keratinocytes, HaCaT keratinocytes also express K1/K10 and α5 integrin [37,43]. The relationship between the expression of these proteins and the proliferation of HaCaT keratinocytes is unexplored. It is also known that serum factors regulate the proliferation and differentiation of cultured human cells. The exact role of serum factors in the regulation of the keratin1, keratin10 and α5 integrin genes is still

unknown. Another marker that is expressed by suprabasal, differentiated keratinocytes is the mannose receptor.

1.2 The skin and the innate immune system

The epithelia constitutes a major barrier to the environment and provides the first line of defense against invading microbes. In the human epidermis keratinocytes represent a physical and biological protective barrier against external pathogens. Keratinocytes have been demonstrated to produce various kind of cytokines, and the epidermis plays an important role in immunologic and inflammatory responses of the body. Keratinocyte-derived cytokines are pivotal in mobilizing leukocytes from blood and signaling to other cutaneous cells.

In addition to regulating immunologic and inflammatory responses, epidermal keratinocytes also contribute to the protective barrier of the epithelia and participate in the first line of defense by killing invading microorganisms. Keratinocytes have been shown to produce inducible antibacterial and antifungal products such as nitric oxide (NO) [44,45,46], antileukoprotease [48,50], LL-37 [49] and beta defensins [47,51]. Keratinocyte-derived NO and antimicrobial peptides in the epidermis could be responsible for killing invading pathogens in the epidermis and preventing systemic invasion of microbes.

Mononuclear phagocytes are believed to play an important role in combating fungal infections and the yeast can be internalized by macrophages though mannose receptor [52] which recognize mannan, the major component of Candida cell wall.

The fact that the removal of the epidermis by scraping prior to inoculation with yeast results in *Candida* pseudohyphae invasion of the dermis also indicates that suprabasal keratinocytes play an important role in the defense against cutaneous *Candida* infection [53]. In both experimental and naturally occurring cutaneous Candida albicans infections the infecting *Candida albicans* are confined to the upper portion of the epidermis [54,55,56]. The majority of the fungal cells are found among epithelial cells of the stratum corneum and they could not be detected in the noncornified cells of the malphigian layer [55].

It has previously been shown that epidermal cells have direct candidacidal activity and this activity can be increased through stimulation of epidermal cells by UV light [57,58], α-melanocyte stimulating hormone (α-MSH) [59] and interleukin-8 [54]. IL-1, prostaglandin E2

(PGE2) and platelet-activating factor (PAF) has also been demonstrated to be involved in Candida killing by human epidermal cells [60] but the mechanism of killing remains unknown.

Human squamous carcinoma cells (SCL-1) and multiply passaged epidermal keratinocytes from normal skin express sugar-binding proteins on their surface [61,62]. It has been also shown that human keratinocytes possess the ability to synthesize and express cell surface moieties characteristic of effector and/or accessory cells of the immune system [63,64]. Using radioligand-binding assay with ^{125}I-mannose-BSA we previously demonstrated that epidermal keratinocytes possess mannose-binding receptors on their surface (Szolnoky et al, in press). Immunohystological staining of normal human epidermis with polyclonal antibody specific for human macrophage mannose receptor showed a membrane staining on suprabasal keratinocytes while the staining with the monoclonal antibody raised against the human macrophage mannose receptor was negative. These results indicate the presence of a mannose binding receptor on the surface of suprabasal keratinocytes and that there is a homology between the putative keratinocyte and the already characterized macrophage mannose receptor (Szolnoky et al, in press).

2. AIMS

Our aims were:

To explore the significance of the expression of α5 integrin, keratin 1 and keratin 10 mRNAs and proteins in the regulation of cell proliferation and differentiation in HaCaT keratinocytes.

To investigate the role of serum factors in the regulation of keratin 1, keratin 10 and α5 integrin genes.

To investigate the specific roles of D1 and D2 type cyclins in the regulation of G0/G1/S transition in keratinocytes.

To demonstrate the presence of mannose binding receptors on normal human keratinocytes.

3. MATERIALS AND METHODS

3.1 Cells

3.1.1 Separation of human epidermal keratinocytes

Human epidermal cells were obtained from healthy individuals undergoing plastic surgery. After removal of the subcutaneous tissue and much of the reticular dermis, the tissue samples were cut into small strips and incubated with dispase solution (Grade II, Roche Molecular Biochemicals) overnight at 4°C. Subsequently, the epidermis was peeled off from the dermis. The epidermis was incubated in 0.25% trypsin solution (Sigma, Csertex, Hungary) at 37°C for 30 minutes and aspirated using a Pasteur pipette to aid cell dissociation. The epidermal cell suspension was filtered through a 100 μm cell filter (BioDesign Inc. of New York, Carmel, NY). Epidermal cell concentration and percentage of viability was determined by hemocytometer. The viability of the cells was always over 95% as determined by trypan blue exclusion. The separated cells were characterized by immunohistochemistry on cytospin preparations (Cytospin, Shandon-Elliot, Frankfurt, Germany). Immunohistochemical analyzes demonstrated that over 95% of the separated cells were keratinocytes and the rest were contaminating melanocytes, no endothelial or Langerhans cells were detected.

3.1.2 Culturing of normal human epidermal keratinocytes

A suspension of primary epidermal cells was prepared in Keratinocyte Serum Free Medium (Keratinocyte-SFM, Gibco, Csertex, Hungary) supplemented with 100 U/ml penicillin and 100 mg/ml streptomycin (each from Gibco). Epidermal cells were seeded into 75 cm^2 tissue culture flasks (Costar, Cambridge, MA, USA) at a density of $4x10^4$cells/cm^2 in Keratinocytes-SFM. Human epidermal keratinocytes were cultured in Keratinocyte-SFM in a humidified atmosphere containing 5% CO_2. The medium was changed every 2 days.

3.1.3 Culturing of HaCaT keratinocytes

Human HaCaT keratinocytes (kindly provided by Dr. N. E. Fusenig, Heidelberg, Germany) were grown in 75cm^2 cell culture flasks (Costar, Cambridge, MA, USA) and

maintained in high glucose Dulbecco's modified Eagle's medium (high glucose DMEM, Gibco, Eggstein, Germany) supplemented with 10% fetal bovine serum (FBS) (Gibco), L-glutamine, penicillin/streptomycin and fungizone (Sigma, Budapest, Hungary) at 37°C in a humidified atmosphere containing 5% CO_2. The medium was changed every 2 days.

3.2 Synchronization procedure

HaCaT keratinocytes were synchronized by cultivating them at high density in the absence of serum. Cells were grown to 100% confluence in medium containing 10% FBS for 5 days, then the medium was replaced by serum-free high glucose DMEM. The cells were grown in serum free media for 1 week. The synchronized cells were trypsinized and were subsequently seeded into 75cm^2 culture flasks at a density of 5x10^3 cells/cm^2 in 10% FBS high glucose DMEM. Samples for propidium iodide (PI) staining, reverse transcription polimerase chain reaction (RT-PCR) and Western blot analyzes were taken from parallel cultures at different times after passing the cells to the 10% FBS containing high glucose DMEM.

3.3 Immunocytochemical analyzes

3.3.1 Immunocytochemistry on cultured HaCaT keratinocytes

Serum starved, confluent HaCaT keratinocytes were passaged into slide chambers (Nunc A/S, Roskilde, Denmark) at a density of 4x10^4 cells/cm^2 The cells were grown in these chambers for different times and the medium was changed every 2 days. For immunocytochemical staining, the cells were fixed for 20 min at 4°C in 2% paraformaldehyde (Sigma) then incubated overnight at 4°C in a humid chamber with the primary antibodies. Mouse monoclonal antibodies specific for human α5 integrin at 1:200 dilution (Clone: SAM-1; Immunotech, Praha, Czech Republic) and mouse monoclonal antibodies specific for human K1 and K10 at 1:100 dilution (ICN, Budapest, Hungary) were used as primary antibodies. Mouse IgG1 served for isotypic control staining. Immunostaining

was performed using an avidin-biotin immunoperoxidase kit (Vectastain Elite kit, Vector Laboratories, Burlingame, CA, USA) with 3-amino-9-ethylcarbazol (AEC; Sigma) as the chromogen. The slides were counterstained with hematoxylin.

3.3.2 Immunocytochemistry on cultured human epidermal melanocytes

Separated human epidermal melanocytes were seeded (Cytospin, Shandon-Elliot, Frankfurt, Germany) onto glass slides. Cells were fixed in acetone (10 min, 4°C) and stained with the anti human MEL-5 mouse monoclonal antibody. Mel-5 is pigment-associated glycoprotein, which is specific for melanocytes. Mouse IgG1 was used for isotypic control staining. The incubation step with goat the anti human MEL-5 (1:1000 dilutions) and the control (1:1000) for overnight at 4°C was followed with incubation of biotin-conjugated secondary antibodies, then streptavidin-biotin peroxidase complex using the Vectastain ABC kit and the instructions of the manufacturer (Vectastain Elite kit, Vector Laboratories, Burlingame, CA, USA). 3-amino-9-ethylcarbazole (AEC, Sigma) was used as a peroxidase substrate. Cells were counterstained with haematoxylin.

3.3.3 Immunocytochemistry on cultured human keratinocytes

Separated keratinocytes were seeded into slide chambers (Nunc A/S, Roskilde, Denmark) at a density of $4x10^4$cells/cm^2 in Keratinocyte-SFM and were maintained in a humidified atmosphere containing 5% CO_2 for different times. For immunocytochemical staining, the cells were fixed for 20 min at 4°C in 2% paraformaldehyde (Sigma), then incubated overnight at 4°C in a humid chamber with the primary antibodies. Mouse monoclonal antibodies specific for human cyclin D1 at 1:250 dilution (PharMingen, San Diego, CA, USA) and mouse monoclonal antibodies specific for human cyclin D2 at 1:250 dilution (PharMingen, San Diego, CA, USA) were used as primary antibodies. Mouse IgG1 served for isotypic control staining for cyclin D1, and mouse IgG2a served for isotypic control staining for cyclin D2. Immunostaining was performed using an avidin-biotin immunoperoxidase kit (Vectastain Elite kit, Vector Laboratories, Burlingame, CA, USA) with 3-amino-9-ethylcarbazol (AEC; Sigma) as the chromogen. The slides were counterstained with hematoxylin.

3.4 Western blot analyzes

Total protein extracts were prepared from synchronized HaCaT cells at different times after the end of the synchronization process and from separated epidermal keratinocytes in a lysis buffer of 1.5% sodium dodecyl sulfate (SDS), 62.5 mM Tris-HCL pH 6.8, 5mM ethylenediamine tetraacetic acid (EDTA), 5% 2-mercaptoethanol (2-ME), 1μg/ml antipain, 1μg/ml chymostatin, and 1μg/ml leupeptin (all chemicals were obtained from Sigma). Lysates were precleared by centrifugation and supernatants were stored at –20°C. The concentration of proteins were defined by UV_{280} absorbtion. The constituent proteins of the keratinocyte extracts were separated by Sodium-Dodecyl-Sulphate-Polyacrylamide-Gel-Electrophoresis (SDS-PAGE) with 9% separating gel (SDS-PAGE) with 9% separating gel and then transferred to nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA). In order to verify the equivalent loadings of proteins in the wells, the gel and the nitrocellulose were stained by Coomassie Brillant Blue and Ponceau S, respectively (Sigma). Membranes were blocked by incubation in Tris buffered saline (150 mM NaCl, 25 mM Tris pH 7.4) containing 0.05% Tween 20 (Sigma) and 3% nonfat dry milk (Fluka Chemie AG, Neu-Buchs, Switzerland) for 2 hours at room temperature and subsequently incubated overnight at 4°C with the appropriate dilution of primary antibodies (mouse monoclonal anti-human α5 integrin (Transcduction Laboratories) diluted 1:250, mouse monoclonal anti-human K1/K10 (ICN) diluted 1:400 and a 1:200 dilution of goat antiserum for human MMR (kindly provided by Prof. Philip D. Stahl), anti human cyclin D1 and D2 (Pharmingen) diluted 1:500) in blocking buffer. Alkaline phosphatase conjugated goat anti-mouse IgG (Sigma) was used as secondary antibody at 1:2500 dilution in the blocking buffer for 2 h at room temperature. Blots were developed by using 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium as a substrate (BCIP/NBT, Sigma).

3.5 Propidium iodide (PI) DNA analyzes

HaCaT cells were harvested by trypsinization and washed in PBS twice. The cell density was adjusted to $1,5 \times 10^6$ cells/ml then 1 ml cell suspension was centrifuged at 1000 rpm for 10 min. The pellet was suspended in 70% cold ethanol (-20°C) and fixed for at least

24 hours at 4°C. Then cells were centrifuged at 3000 rpm and were suspended in 1 ml propidium iodide staining buffer (50μg/ml PI, and 100U/ml RNase A, all from Sigma) and stained for 30 min at room temperature. The samples were analyzed using Facscalibur (Becton Dickinson) and Modfit (Verity Software House, Inc., Topsham, ME, USA).

3.6 Flow cytometric analyzes of separated epidermal keratinocytes

$1x10^6$ freshly separated human keratinocytes were incubated with 100 ml goat anti-human MMR antiserum dissolved in 0.5% BSA-PBS at a dilution of 1:25 for 1 h on ice. For isotype control preimmune goat serum was used at a dilution of 1:25. Cells were washed twice in PBS and incubated for another hour on ice with biotinylated mouse anti-goat IgG at 1:200 dilution. After washing again twice in PBS cells were incubated with 100 ml streptavidin-PE (Becton Dickinson, Heidelberg, Germany) at 1:100 dilution for 1 h on ice. Cells were washed twice, then fixed in 1% paraformaldehyde and analyzed by flow cytometry using a FACStar Plus Flow Cytometer (Becton Dickinson). Data (10000 events per sample) were analyzed using the Cell Quest 3.1 F program. To investigate the trypsin sensitivity of the mannose binding receptors cells were incubated with 10 mg/ml trypsin (type I from bovine pancreas, Sigma) for 10, 60 or 120 min prior to binding assay.

3.7 Reverse transcription polimerase chain reaction (RT-PCR)

Total RNA was isolated from $1x10^6$ HaCaT cells by TRIzol^{TM} reagent (Gibco) following the instructions of the manufacturer. First strand cDNA was synthesized from 3 μg of total RNA in 20 μl final volume using a First Strand cDNA Synthesis Kit (MBI Fermentas, Vilnius, Lithuania). After reverse transcription, amplification was carried out by PCR reaction using the Taq DNA Polymerase and dNTP Set of MBI Fermentas. 10 μl of the reverse transcription volume was used as a template for α5 integrin specific PCR, 5 μl for tyrosinase0,5 μl of the reverse transcription reaction was used for K1 and K10 keratin specific PCR, 2μl of the reverse transcription reaction was used for cyclin D1 specific PCR and 1μl of the reverse transcription volume was used as a template for the β actin specific PCR. Specific

primers listed in table 1 were included in the reactions at 0.66 pmol/μl final concentration. The PCR reactions amplified a 358 bp long product for α5 integrin, a 316 bp for keratin 1, a 685 bp for keratin 10, a 162 bp product for cyclin D1, 198 bp for tyrosinase and a 406 bp for β-actin. The same PCR conditions were used for all PCR reactions: 94 °C 90 sec., 60 °C 90 sec., 72 °C 120 sec. The number of cycles were as follows: 35 cycles for α5 integrin, cyclin D1 and tyrosinase, 27 cycles for K1 and K10 keratin, and 25 cycles for β-actin. The concentration of $MgCl_2$ was 1.5 mM in all PCR reactions. The yielding products were run on 2% agarose gel, stained with ethydium bromide, photographed and evaluated by Kodak Edas120 densitometer and Kodak 1D Digital Science software and (Scientific Imaging Systems, New Haven, CT, USA).

Primers	Forward	Reverse
α5 integrin	ATTATCAGAGCAAGAGCCGGATAGA	GGAGATGAGGGACTGTAAACCGA
Keratin 1	GGACATGGTGGAGGATTACCG	TGCTCTTCTGGGCTATATCCTCG
Keratin 10	GCAAAATCAAGGAGCGGTATGA	GAGCTGCACACAGTAGCGACC
Cyclin D1	AGGAGAACAAACAGATCA	TAGGACAGGAAGTTGTTG
Tyrosinase	TTGGCAGATTGTCTGTAGCC	AGGCATTGTGCATGCTGCTT
β-actin	AGAGATGGCCATGGCTGCTT	ATTTGCGGTGGACGATGGAG

3.8 Silver staining of proteins in SDS polyacrylamide gel

A mannose binding protein was purified from human epidermal keratinocytes receptor by affinity chromatography on mannose coupled epoxy activated mannose-Sepharose 6B column (Pharmacia Biotech, Uppsala, Sweden). The purified mannose-binding protein was separated by Sodium-Dodecyl-Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) with 7.5% separating gel under non-reducing conditions. After electrophoresis, proteins were fixed by incubating the gel in a solution of ethanol:glacial acetic acid:water (30:10:60) for overnight at 4°C. Then the gel was washed in 50% ethanol and deionized water. After the last washing step the gel was incubated in a 0,1% solution of $AgNO_3$ for 30 minutes at room temperature. Then the gel was washed in deionized water and incubated in aqueous solution

of 2,5% sodium carbonate, 0,02% formaldehyde at room temperature with gentle agitation until stained bands of the protein appeared. The reaction was quenched by washing the gel in 1% acetic acid.

4. RESULTS

4.1. Expression of α5 integrin, keratin 1 and keratin 10 correlates with the proliferation of HaCaT keratinocytes

4.1.1 Expression of α5 integrin in HaCaT keratinocytes is regulated by environmental conditions

In order to achieve homogeneity of HaCaT keratinocytes the cells were synchronized. To avoid the use of chemical treatments, HaCaT keratinocytes were synchronized by cultivating the cells at high density in the absence of serum for one week. Then we forced the cells to re-enter the cell cycle by passing them into 10% FBS containing media. The expression of α5 integrin both at the mRNA and protein levels was examined at 0, 12, 24, 36, 48, 72, 96 and 168h following the end of the synchronization process using RT-PCR, Western blot analyzes and immunocytochemistry. The state of the synchronized culture at different times was also observed by morphology (Fig. 2). A very low level of α5 integrin mRNA expression was detected at 0h with RT-PCR (Fig. 1a). The level of α5 integrin mRNA raised 12h after release from quiescence (Fig. 1a) and afterwards its level gradually increased (Fig. 1a 24h, 36h) until it reached a maximum level at 48h (Fig. 1a). The α5 integrin mRNA level remained high until 96h (Fig. 1a). This high-level expression coincided with the intensive proliferation of the cells (Fig. 2). Before the culture reached confluence, the expression of α5 integrin mRNA started to decrease (Fig. 1a, 168h). The level of α5 integrin protein showed a very similar kinetics, but as expected, the raise of the α5 mRNA preceded that of the protein. The α5 integrin protein was undetectable in the synchronized, serum starved cells at 0h (Fig. 1b, 0h), then its amount started to rise gradually and reached its maximum level at 96h (Fig. 1b). By 168h, the α5 integrin level has decreased considerably (Fig. 1b). The α5 integrin expression was also examined by immunocytochemistry to see the differences in the expression among individual cells. α5 integrin was similarly expressed in all cells (Fig. 3a) with the exception of mitotic cells, in which α5 integrin expression appeared to be stronger (Fig. 3a, arrows). In the immunocytochemical analyzes, similar to the Western analyzes, the

α5 integrin expression was highest before the cells started to become confluent and afterwards, at higher cell density, the expression faded (Fig. 3b). Although one would expect a more distinct membrane localization, in the very active proliferative state of HaCaT cells the large amount of newly synthesized protein is localized to the cytoplasm (Fig. 3a). Interestingly, in the confluent state of the culture when the protein level is much lower (almost undetectable signal at 168h in the Western blot analyzes; Fig. 1b) one could clearly see the membrane localization of the protein (Fig. 3b).

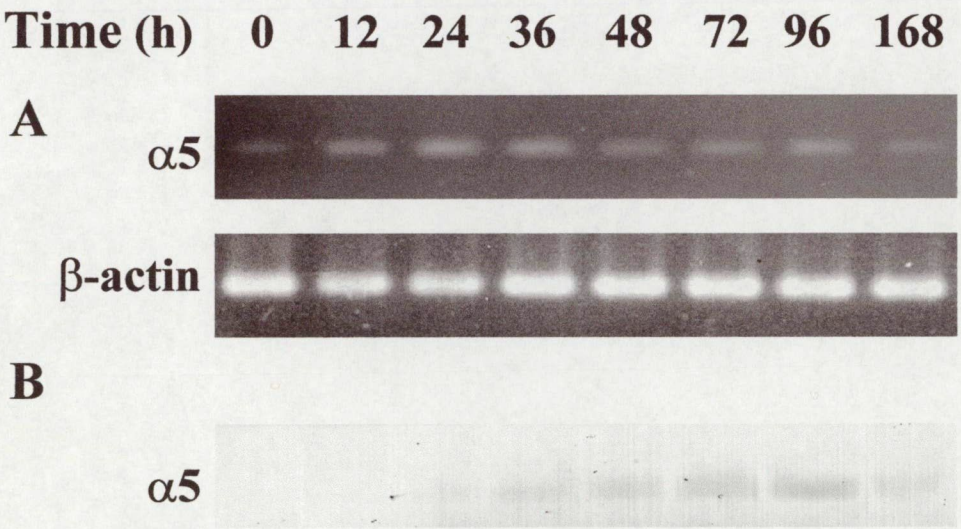

Figure 1. α5 integrin expression in HaCaT keratinocytes after release from quiescent state. The expression of α5 integrin was analyzed by RT-PCR (a) and by Western blot analyzes (b) at the times indicated after releasing the cells from serum starvation and contact inhibition.

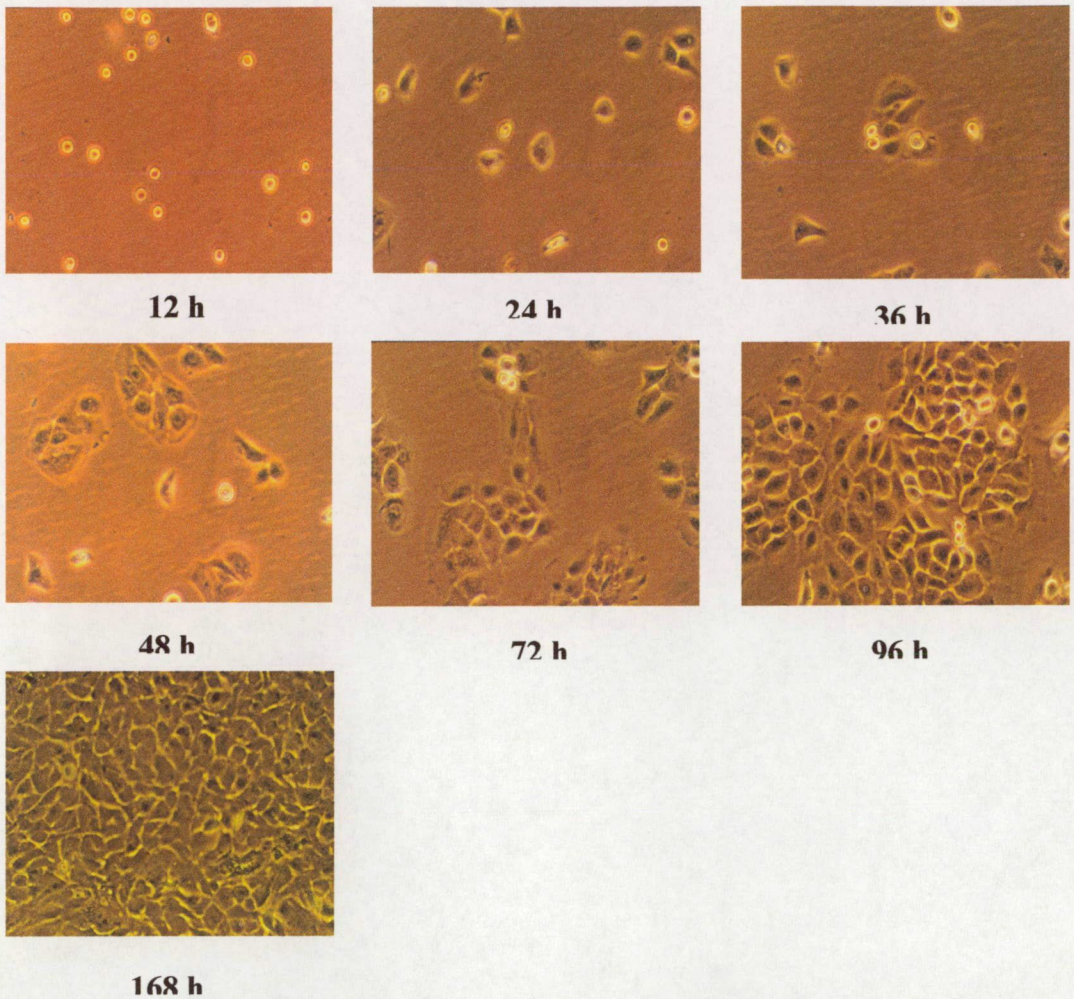

Figure 2. Morphological changes in the synchronized HaCaT culture different times after release from cell quiescence.

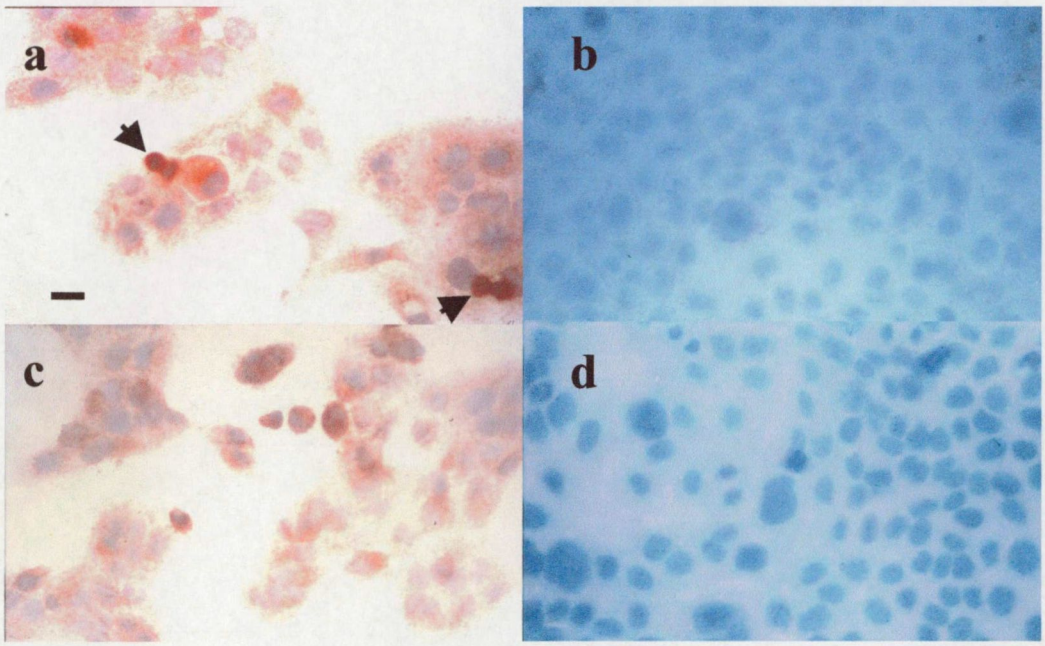

Figure 3. Immunocytochemical staining of HaCaT keratinocytes for α5 integrin and K1/K10 at different times after release from cell quiescence. 36 h after serum addition and passage all cells in the culture show α5 integrin staining (a). Mitotic cells in the culture (arrows) appear to have a stronger α5 integrin expression relative to the other cells (a). Later, when the cells reach higher density, the α5 integrin expression decreases in the cells (b). HaCaT keratinocytes stain strongly for K1 and K10 shortly after release from cell quiescence (c). Later, in the more confluent culture, the expression of K1 and K10 decreases. Bar = 50μm.

4.1.2 HaCaT keratinocytes lose K1/K10 expression after release from synchronized state

The expression of K1/K10 was examined in the same system. Both K1 and K10 mRNAs were highly expressed in the synchronized, serum depleted and contact inhibited HaCaT keratinocytes (Fig. 4a, 0h). After release from quiescence, the level of K1 and K10 mRNAs decreased dramatically. A very low level of K1 mRNA was still detectable at 12h in the culture, but the mRNA level of keratin 10 became undetectable in the 12h sample. K1 and K10 mRNAs were undetectable after the 12h (Fig. 4a). Changes in the levels of K1 and K10 proteins were followed by Western blot analyzes. K1 and K10 proteins were present in the cells in extremely large amounts in the synchronized, serum depleted culture (Fig. 4b,

0h). Afterwards both K1 and K10 protein levels started to decrease very slowly (Fig. 4b, 12h, 24h, 36h, 48h, 72h), but were still high even 72h after the end of synchronization, despite the apparent lack of new protein synthesis during this period. K1 protein became undetectable by 96 h (Fig. 4b), at the same time K10 protein was still detectable even in the 168h sample (Fig. 4b), although its amount has decreased considerably. The expression of K1/K10 keratins were also examined by immunocytochemistry to make certain that the decrease in the levels of K1/K10 proteins detected by Western analyzes was not due to differential expression by a subpopulation of cells within the culture. Individual cells did not show differences in K1/K10 expression and the expression kinetics observed by immunocytochemistry was very similar to the Western analyzes. The level of K1/K10 proteins decreased dramatically from the initial high level (Fig. 3c) to a very low level as the culture moved toward confluence (Fig. 3d). The above-described kinetics of K1/K10 mRNA and protein expressions in HaCaT cells indicate that both keratin types are very long-lived proteins. In fact there is no evidence in normal keratinocytes that K1/K10 once synthesized could disappear from the cells. The ability to degrade K1/K10 may be a special characteristic of HaCaT keratinocytes and could be a key function in their immortality.

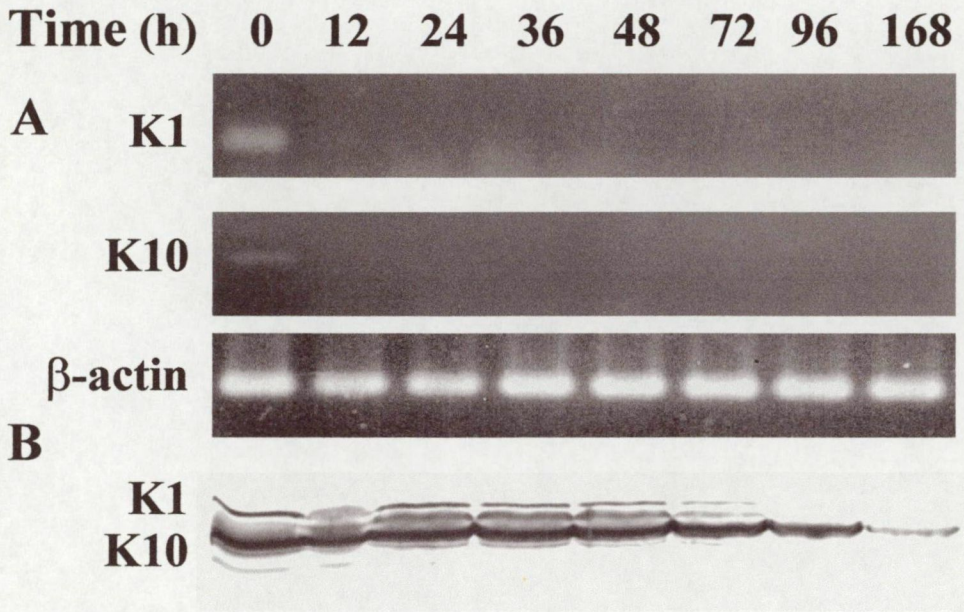

Figure 4. K1 and K10 expression in HaCaT keratinocytes after release from cell quiescence. Changes in the expression of K1/K10 mRNAs were analyzed by RT-PCR (a), K1/K10 proteins were analyzed by immunoblotting (b) at the times indicated after release from cell quiescence.

4.1.3 α5 integrin mRNA and protein expression increase while keratin 1 and 10 mRNAs and protein decrease dramatically with proliferation in HaCaT keratinocytes

D1 cyclin mRNA expression and DNA staining with PI were used to follow the proliferation state of the synchronized HaCaT cells after release from contact inhibition and serum starvation. Cyclin D1 is an early G1 phase marker [65]. After 1 week of serum starvation the cells had undergone an almost complete cell cycle withdrawal. At 0h both the cyclin D1 mRNA (Fig. 5) and the number of S/G2/M phase cells (Fig. 6a) were very low in the culture and they both remained at low level during the first 24h after release from quiescence. A gradual increase in cyclin D1 message was detected between 24 and 72h (Fig. 5) that was closely followed by an increasing number of cells entering S phase (Fig. 6), indicating a high rate of proliferation among the cells by day 3. K1/K10 mRNAs decreased to undetectable levels between 24 and 48h (Fig. 6b). Although the cells still contained high levels of K1/K10 proteins, there was a substantial relative decrease for both keratins by day 3 (24,3% for K1 and 46,2% for K10, Fig. 6c). A dramatic increase in α5 integrin mRNA preceded the high level proliferation of the cells at 24 h (Fig. 6b), that was followed by a gradual increase in α5 integrin protein expression (Fig. 6c). The increase in the amount of α5 integrin protein paralleled the increase in the number of S/G2/M phase cells (Fig. 6a and c), indicating a close connection between α5 integrin protein expression and cell proliferation.

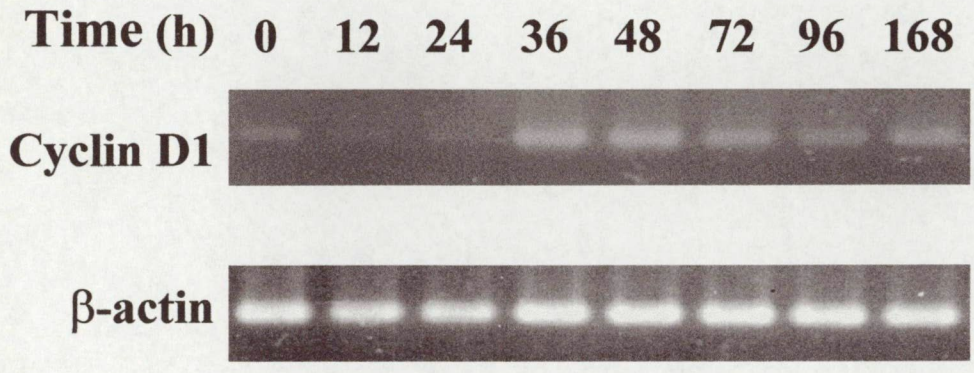

Figure 5. The expression of cyclin D1 mRNA in HaCaT keratinocytes after release from cell quiescence.

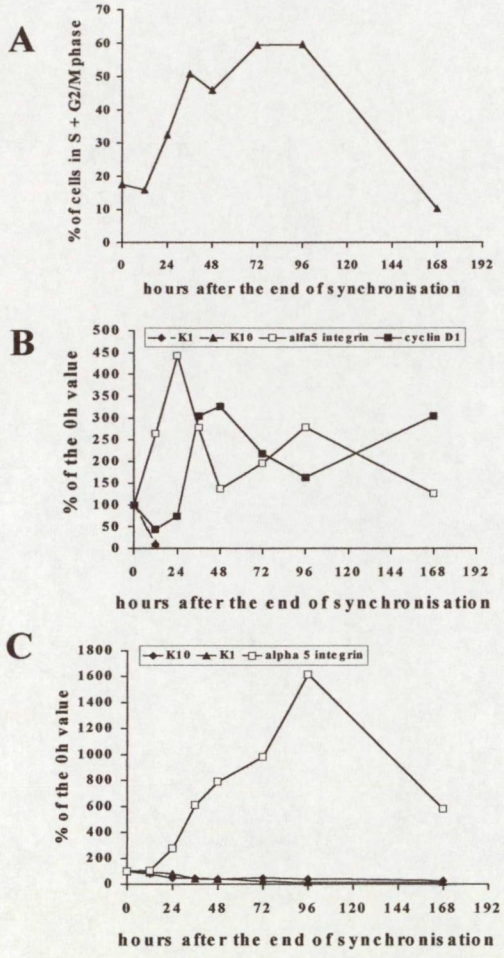

Figure 6. Comparison of cell proliferation and the expression of α5 integrin, K1 and K10 in HaCaT cells.

4.1.4 The roles of cyclin D1 and D2 are different in human keratinocytes

To establish whether keratinocytes in different stages of differentiation require different subsets of these complexes, we analyzed the expression of cyclin D1 and D2 in cultured keratinocytes. Freshly separated human epidermal keratinocytes from healthy adults were seeded onto slide flasks. Previously it was demonstrated that cultured human keratinocytes represented the K1/K10- basal cells 24 hours after seeding, which entered the G1/S phase from G0 quiescent state (G0/G1/S transition). 72 hours after seeding, cultured keratinocytes have completed the first cell division and entered the next G1/S phase (they

represented the transiently amplifying cells within the epidermis [19]. Immunocytochemical staining was performed on the cultured keratinocytes 24 and 72 hours after seeding with monoclonal anti human cyclin D1 and D2 antibodies. We found that 24 hours after seeding when keratinocytes entered the cell cycle from quiescence (G0 phase), cells showed a positive nuclear staining for cyclin D1 (Fig. 7a) but not for cyclin D2. 72 hours after seeding, proliferating keratinocytes that entered from G1 but not from G0 into S phase, showed a positive nuclear staining for cyclin D2 (Fig. 7b) but not for cyclin D1, indicating a different role for D1 and D2 cyclins in the regulation of cell cycle progression. Interestingly, in some of the cells cyclin D2 showed cytoplasmic staining (Fig. 7c) which was never observed for cyclin D1.

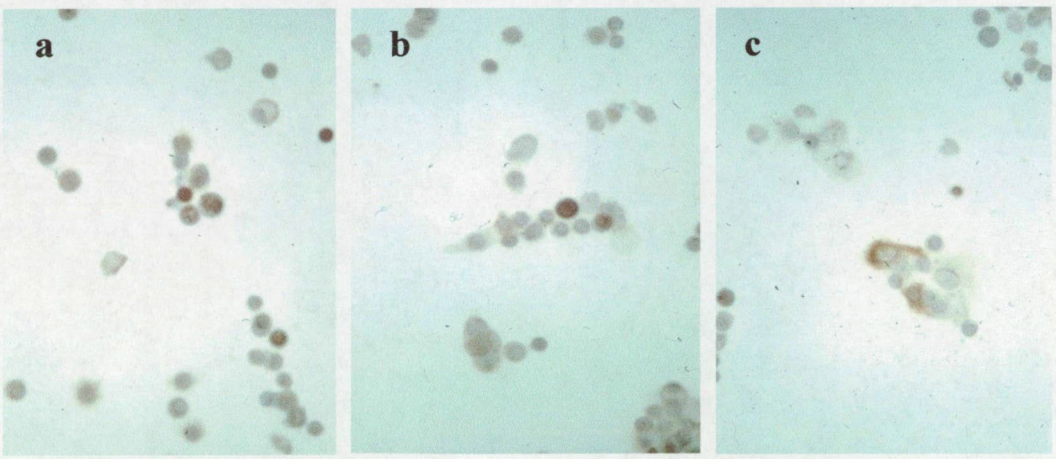

Figure 7. Immunocytochemical staining of cultured human keratinocytes for cyclin D1 (a) and D2 (b and c) at different times after seeding.

4.1.5 Expression of cyclin D1 and D2 in cultured human epidermal melanocytes

To investigate whether the differential expression of D-type cyclins is typical only to keratinocytes or also to other cell types, the expression of cyclin D1 and D2 was investigated in human epidermal melanocytes with Western analyzes. We found that cyclin D1 was predominantly expressed in proliferating melanocytes in every passages although cyclin D2

protein expression was also observed in a much less extent. (Fig. 8)

To gain melanocytes epidermal cell suspension was seeded onto tissue culture flasks. The primary cell culture still contained both keratinocytes and melanocytes. Keratinocytes were progressively removed from the culture during culturing and pure melanocyte cultures were obtained. This separation is based on the different attachment characteristics of keratinocytes and melanocytes to the culture plastic flask. Trypsin treatment release melanocytes 2-3 minutes earlier than keratinocytes from attachment to the tissue culture plastic, thus enabling separation of the two cell populations. Melanocytes were identified by morphological characteristics, tyrosinase mRNA expression and a melanin-associated glycoprotein (MEL-5) expression that were performed on melanocytes at different passage numbers (Fig. 9).

The expression of tyrosinase is a specific marker of melanocytes. Expression of the tyrosinase gene in cultured cells was examined by RT-PCR (Fig. 9c). The results showed that cells expressed tyrosinase mRNA at high level up to the 10^{th} passage, confirming their melanocyte origin. In subsequent cultures the expression of tyrosinase started to decrease to a very low level (Fig. 9c). Cultured cells showed typical melanocyte morphology (Fig. 9a) and were positively stained with MEL-5 antibody (Fig. 9b) verifying their melanocyte origin. The MEL-5 staining was detectable usually up to the 5^{th} passage, showing a good correlation with the presence of melanin in the cells. As it was expected, the expression of tyrosinase and the positive staining with MEL-5 showed a correlation with the pigment content of cultured melanocytes.

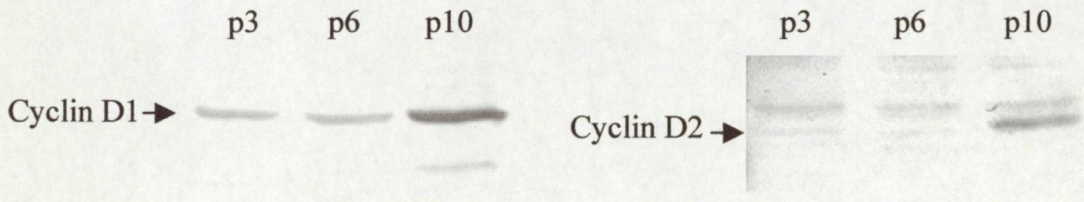

Figure 8. Expression of cyclin D1 and D2 proteins was analyzed by Western blot analyzes in proliferating epidermal melanocytes in different passages.

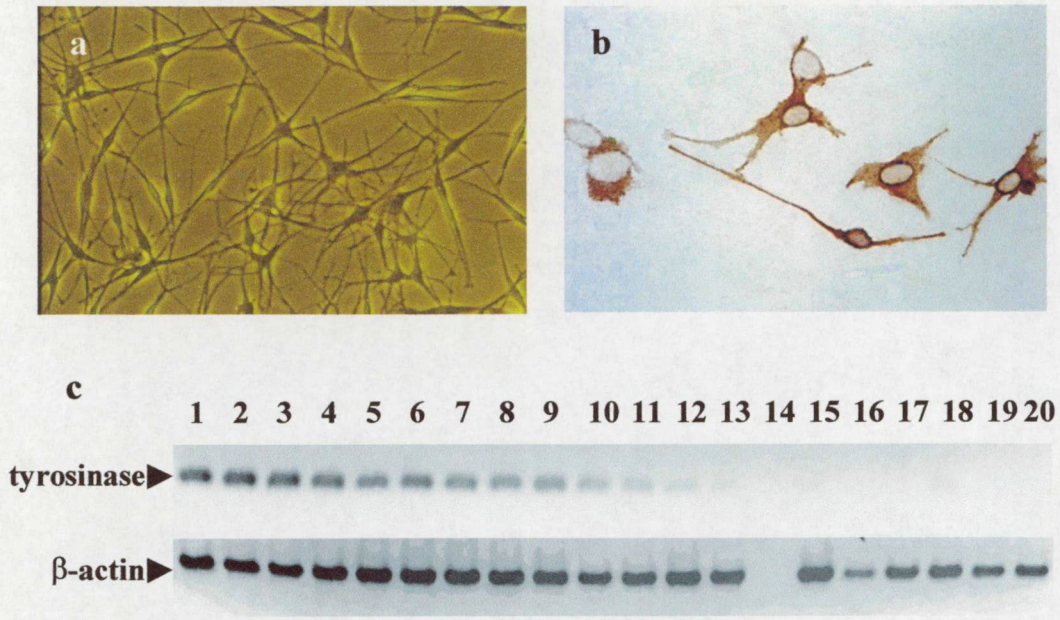

Figure 9 Identification of melanocytes. Cultured cells showed dendritic or spindle-like morphology, which is typical for melanocytes (a). Cultured cells were positively stained with melanocyte-specific antibody MEL-5 (b). Melanocytes expressed tyrosinase mRNA up to the 13th passage (c).

4.2. Serum factors regulate the expression of keratin 1 and α5 integrin genes but not the keratin 10 gene in HaCaT keratinocytes

To see whether the dramatic down-regulation of K1/K10 gene expression and the significant up-regulation of α5 integrin mRNA expression were driven by serum factors or contact inhibition, RT-PCR analyzes was used. The effect of serum deprivation was examined on freshly seeded HaCaT cells to exclude the effects of contact inhibition on the expression of these genes. These cells were not starved and synchronized prior to seeding. Seeding was performed in the presence of serum and the cells were kept in the serum-supplemented media for 12h before serum withdrawal. 12h after seeding, only a very low level of K1 and K10 expression could be detected in the cells (Fig. 10, 12h). 24h after serum withdrawal, HaCaT keratinocytes showed a dramatic increase in K1 mRNA expression (Fig. 10, 36h), suggesting a strong suppression of the K1 gene by serum factors. Later, at 48 and 72h after serum

withdrawal, the expression of K1 mRNA decreased gradually (Fig. 10, 60 and 84h), indicating that additional regulatory mechanisms also exist. Surprisingly, the K10 mRNA level remained unchanged upon serum withdrawal (Fig. 10), indicating that the expression of keratin 10 gene is not regulated by serum factors. A relatively strong α5 integrin mRNA expression was found at 12h in the culture in the presence of serum (Fig. 10, 12h). After removal of the serum from the culture media, the α5 integrin mRNA expression decreased to an almost undetectable level (Fig. 10, 36, 60h), suggesting that serum factors regulate the α5 integrin gene expression. 72h after serum withdrawal the expression of α5 integrin mRNA reappeared in the cells (Fig. 10, 84h), indicating that beside serum factors other regulatory mechanisms also exist.

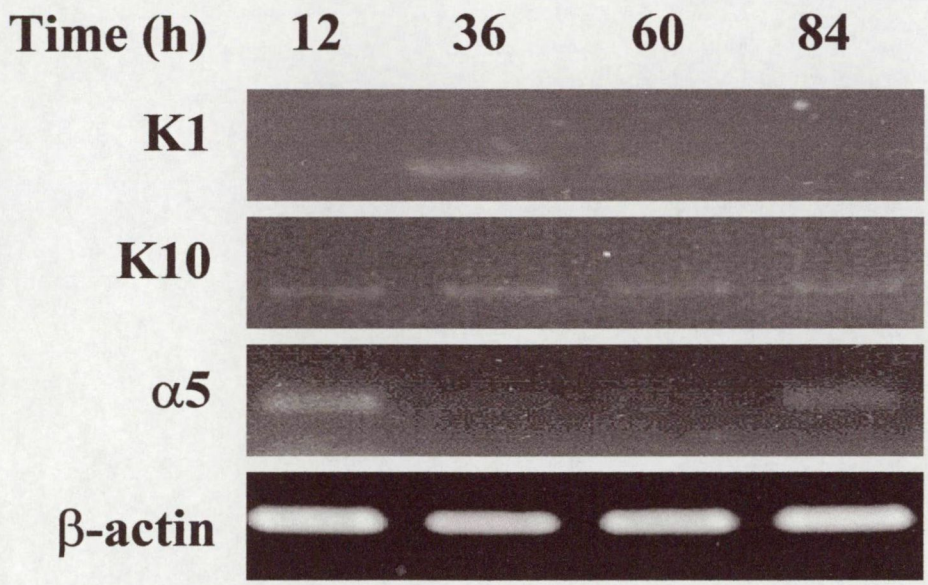

Figure 10. Effect of serum withdrawal on the expression of α5 integrin and K1/K10 mRNAs in HaCaT keratinocytes (RT-PCR).

4.3. Further characterization of the mannose binding receptors expressed on suprabasal human keratinocytes

4.3.1. Flow cytometric detection of mannose receptors on the surface of separated epidermal keratinocytes

To demonstrate that epidermal keratinocytes express mannose-binding receptors on their surface, freshly separated human epidermal keratinocytes were stained with the goat polyclonal sera raised against the human macrophage mannose receptor and analyzed by flow cytometer. Since it is known that macrophage mannose receptor is sensitive to proteolysis we aimed to determine the trypsin-sensitivity of keratinocyte mannose receptor and its recovery after trypsinization. A preliminary experiment with radiolabelled mannose-BSA showed that keratinocyte mannose receptor was sensitive to proteolysis (Szolnoky et al, in press). To explore the time kinetic of the recovery of mannose receptors after trypsinization, freshly separated keratinocytes were incubated in PBS at 37°C for 10, 30, 60 and 120 min after trypsin exposure. Both the ratio of positive cells and the ΔMCF (Δ -mean channel fluorescence = mean channel fluorescence of cells stained with the goat immune serum minus mean channel fluorescence of cells stained with non-immune goat serum) showed a rapid, time-dependent increase in mannose receptor expression on keratinocytes. After the shortest incubation (10 min), 32.5% of the cells already showed positive staining (Fig. 11) relative to the isotype control and the ΔMCF was 8.36, indicating that mannose receptors on keratinocytes can recover very rapidly after trypsinization. As a result of prolonged incubation we detected a time-dependent increase in both the number of mannose receptor positive cells (Fig. 11; 30 min: 49.38%, 60 min: 56.18%, 120 min: 65.81%) and in ΔMCF (30 min: 12.47, 60 min: 14.08, 120 min: 17.05).

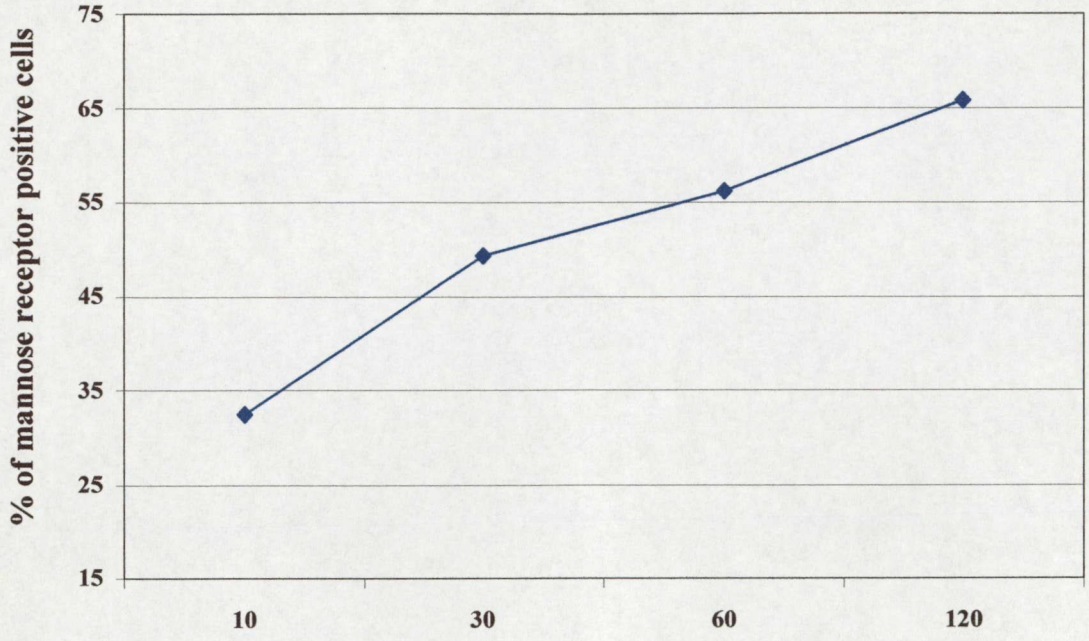

Figure 11. Recovery of keratinocyte mannose receptor after trypsinization.

4.3.2 Western blot analyzes of the keratinocyte mannose binding receptor

Western blot analyzes was also used to demonstrate the presence of the mannose binding receptor on separated epidermal keratinocytes and on the immortalized human keratinocyte cell line HaCaT. On Western blots of human keratinocyte and HaCaT cell extracts the polyclonal goat anti-human MMR antibody stained a band with a molecular weight of approximately 200 kD both in normal keratinocytes and HaCaT cells (Fig. 12).

Figure 12. Western blot analyzes of keratinocyte mannose receptor on normal human keratinocytes and HaCaT cells.

4.3.3 Silver staining of affinity purified mannose receptor

In order to further characterize the keratinocyte mannose receptor we isolated the receptor by affinity chromatography on mannose-Sepharose column from human keratinocyte extract. The eluted protein was concentrated and separated by Sodium-Dodecyl-Sulphate-Polyacrylamide-Gel-Electrophoresis (SDS-PAGE). The protein band visualized by silver staining revealed a single band at approximately 200 kD (Fig. 13).

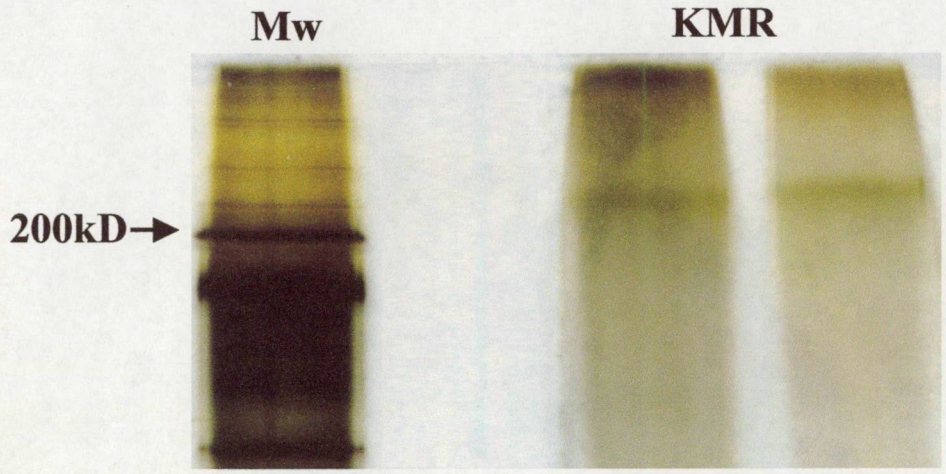

Figure 13. Silver staining of affinity purified keratinocyte mannose receptor.

5. DISCUSSION

The skin is a protective barrier against the external environment. The uppermost layer of the skin is the epidermis, which is a continuously renewing tissue. The most abundant cell type in the epidermis is the keratinocyte. Terminally differentiated corneocytes provide a mechanical barrier, while suprabasal keratinocytes are able to accomplish immunological functions as killing invading microorganisms and alert other immune components in the skin. In the epidermis only the innermost basal layer of keratinocytes has proliferative capacity that makes the epidermis renewing by replacing the continuously loosening corneocytes. Under a yet unidentified trigger of terminal differentiation, a basal keratinocyte stem cell undergoes a series of morphological and biochemical changes as it becomes a fully differentiated corneocyte.

In the present work we investigated two interesting aspects of the human epidermis: regulation of differentiation and proliferation of human keratinocytes and the molecular basis of immunological role of keratinocytes. For investigating the keratinocyte differentiation markers in vitro, we developed a model. In this model, proliferation and differentiation markers of synchronized HaCaT keratinocytes could be investigated in vitro, without the use of chemical treatments. With changing the environmental conditions of cultured cells, we could differentiate them and then induce their de-differentiation. This model provides a tool for investigating epidermal keratinocytes in different states of differentiation and proliferation.

In order to better understand the proliferation and differentiation processes of keratinocytes, we have chosen molecular markers that are characteristics for specific keratinocyte populations. Analyzes of the expression of α5 integrin, K1/K10, and the proliferation state of the synchronized HaCaT cell cultures revealed a close correlation between the proliferation of HaCaT cells and the expression of α5 integrin both at the mRNA and protein levels. The α5 integrin mRNA expression precedes and the α5 integrin protein expression coincides with the intensive proliferation of the cells, defined by the expression of cyclin D1, an early G1 marker, and PI DNA analyzes. These facts suggest that α5 integrin may play a role in the promotion of HaCaT proliferation. This finding is not surprising, since

increased expression of α5 integrin on keratinocytes in the *in vivo* epidermis is associated with hyperproliferation of the cells [13,66]. The *in vivo* significance of α5 integrin expression and function in the epidermis have been demonstrated by forced suprabasal expression of α5 integrin of transgenic mice that resulted in a phenotype reminiscent of the hyperproliferative skin disease, psoriasis [67].

As opposed to α5 integrin, the down-regulation of K1/K10 at the mRNA level seems to be a prerequisite for proliferation of HaCaT cells, indicating an inhibitory role for these keratins in proliferation. Although at the time of intensive proliferation K1/K10 proteins were still present in the cells, new proteins could not be synthesized, since no mRNA was present for these keratin types. It is possible that a continuous synthesis of keratins are required to block cell proliferation. The newly synthesized keratins may differ from the already existing keratins in various functions such as inhibition of proliferation. Changes in keratin functions may be due to posttranslational modifications occurring during differentiation of keratinocytes, as it was shown by Fuchs et al. [6]. Together with actin microfilaments and microtubules, keratin filaments make up the cytoskeleton of epithelial cells. Although these proteins are thought to be involved in maintaining the mechanical integrity of epithelial cells, their function in the complex differentiation process is still unclear. It was shown that ectopic expression of K10 inhibits the proliferation of HaCaT cells in culture [23].

Our results also indicate that the expression of K1 gene is tightly regulated by serum factors and the K1 and K10 genes are regulated differentially. Although it has been previously reported that the expression of K1 and K10 could be regulated independently, the background of this differential regulation was not clarified [37]. Based on the results of our experiments we conclude that the expression of K1 is regulated by serum factors, but other regulatory mechanisms also exist. On the other hand, serum factors seem to have no direct regulatory effect on K10 gene expression.

The expression of α5 integrin is linked to an undifferentiated, intensively proliferating state of HaCaT keratinocytes and α5 integrin is expressed in an opposite way to the K1/K10 keratins. This is similar to the expression of α5 integrin in normal human keratinocytes in which α5 integrin expression coincides with a highly proliferative, undifferentiated state of the cells [10].

We found a strong expression of α5 integrin in HaCaT cells growing in the presence of serum that decreased dramatically after the removal of serum from the culture media, indicating that serum factors have a role in the regulation of α5 integrin gene expression. This finding may be relevant to the situation of wound healing where keratinocytes are exposed to serum factors, that could be responsible for the induction of α5 integrin expression on keratinocytes in the wound. One important factor present in the serum, that is known to regulate α5 integrin expression in various cells is fibronectin [7,13,68-70].

HaCaT keratinocytes under serum free, contact inhibited conditions with the strong expression of K1/K10 and the suppressed expression of α5 integrin resemble the suprabasal, non-proliferating keratinocytes of normal human *in vivo* epidermis. It is possible that serum factors that are able to diffuse through the basement membrane from the dermis into the epidermis regulate the expression of K1 in the *in vivo* epidermis. As keratinocytes move away from the basement membrane, the concentration of these membrane-diffusable serum factors gradually decrease and the cells start to express K1. However, in normal keratinocytes there is no evidence that the expression of K1 and K10 could be reversed. The ability of HaCaT cells to dedifferentiate seems to be a unique feature of these cells and maybe related to their immortalized nature [37].

Our data support previous investigations describing similarities between HaCaT cells and normal keratinocytes and concluding that HaCaT cells are excellent candidates for studying external regulators of proliferation and differentiation and the underlying molecular mechanisms [37]. Our findings also emphasize the importance of the *in vitro* culture conditions of the cells. HaCaT keratinocytes under different conditions represent different types of keratinocytes. In the serum starved, contact inhibited culture HaCaT cells resemble the suprabasal, non-proliferating, differentiated (K1/K10+) keratinocytes of normal epidermis, while the highly proliferative HaCaT cells (α5 integrin+, K1/K10+) after release from contact inhibition and addition of serum resemble the activated (α5 integrin+), differentiated (K1/K10+), transiently amplifying keratinocytes.

As a result of recent studies, the cyclin dependent kinase complex has come to be taken up as a target of proliferation and carcinogenesis of keratinocytes. Cyclins D1, D2 and D3 are thought to function in the G1 phase of the cell division cycle by regulating the activity

of cyclin-dependent protein kinases. All three D-type cyclins can be shown to associate with two specific kinases, CDK4 and CDK6 and to bind to retinoblastoma protein (Rb), providing at least six possible combinations [71]. To establish whether keratinocytes in different stage of differentiation require different subsets of these complexes, we analyzed the expression of cyclin D1 and D2 in keratinocytes. We showed that not only the differentiation and immune associated markers change their expression pattern during this process, but also important cell cycle regulators, cyclin D1 and D2. We hypothesize that basal epidermal keratinocyte stem cells when enter into the cell cycle express cyclin D1 but not D2 in early G1 phase. The proliferating transiently amplifying keratinocytes that are committed to differentiation express cyclin D2 but not cyclin D1 for G1/S transition. As cyclin D1 and cyclin D2 phosphorilate the Rb protein differentially, the different D type cyclins may have different effects on gene regulation and cell proliferation. The difference may be associated with the different functions and fate of keratinocytes in the epidermis. To explore that differential use of type1 and type 2 D cyclins is a unique feature of keratinocytes or typical for other cell types in the epidermis, we investigated the expression of D-type cyclins in cultured epidermal melanocytes. Similarly to keratinocytes melanocytes also use these cyclins differentially during the cell cycle progression.

We showed the presence of a mannose binding receptor on the surface of suprabasal epidermal keratinocytes. This finding also demonstrates that epidermis is not only passive physical barrier but an active immune organ. In this immune organ epidermal keratinocytes are able to kill invading pathogens and prevent their systemic invasion by producing nitric oxide and antimicrobial peptides. The fact that the polyclonal antibody raised against the human macrophage mannose receptor (hMMR) is able to recognize keratinocyte mannose receptor (KMR) indicates a homology between the two receptors. However, the monoclonal anti-human macrophage mannose receptor antibody does not recognize the keratinocyte mannose receptor. This finding indicates that the two receptors are not identical. We showed that the mass of the keratinocyte mannose binding protein is approximately 200 kD while the mass of the macrophage mannose receptor is around 175 kD. We also compared the trypsin sensitivity of the macrophage and keratinocyte mannose receptor. Similarly to the macrophage mannose receptor, the keratinocyte mannose receptor is also sensitive to trypsinization (Szolnoky et al., in press). In the case of macrophage mannose receptor it was

shown that removal of surface expressed MMR activity by trypsin incubation at 4°C was followed by a fast (10 min) recovery of the receptor activity at 37°C suggesting the existence of an intracellular pool of receptors [72]. We determined that the recovery of keratinocyte MR after trypsinization is also a rapid process, as in the case of macrophage MR.

Using affinity chromatography on Mannose-Sepharose column from keratinocyte cell extract, we isolated a singular protein with a molecular weight of ~200 kD. Since the molecular weight of macrophage mannose receptor is only ~175 kD this result together with the finding that the polyclonal anti MMR serum but not the monoclonal anti MMR could recognize the KMR in the epidermis suggest that KMR is homologus but not identical to MMR. Using Western analyzes with the polyclonal anti human macrophage mannose receptor antibody a specific band was found with an apparent molecular weight of 200 kD.

Based on our data (Szolnoky et al., in press), keratinocyte macrophage mannose receptor does not mediate phagocytosis of pathogens and the mechanism of killing invading microorganism may be different in keratinocytes and macrophages. In rat neutrophils, nitric oxide (NO) was shown to be involved in the candidacial activity [73]. Since human keratinocytes are also able to express inducible nitric oxide synthase (iNOS) NO is likely to act as a key component in the first line of defense in the skin. Our group has demonstrated that NO produced by keratinocytes also plays a crucial role in the *Candida* killing mechanism. Freshly separated keratinocytes isolated from iNOS knockout mice were not able to kill *Candida albicans*, while the keratinocytes isolated from wild-type mice show Candidacial activity like human keratinocytes (unpublished results). We hypothesize that keratinocyte mannose receptor may be involved in the induction of the expression of iNOS by invading pathogens. The activation of mannose receptor may result in the activation of iNOS gene expression and NO production. The production of NO by epidermal keratinocytes could be responsible for killing various bacteria, and fungi, which have been shown to be sensitive to NO such as *Candida albicans*, *Mycobacterium tuberculosis* and *Escherichia coli* [74].

Recently, several broad spectrum microbicidal molecules have been identified in mammalian epithelium that are inducible or expressed constitutively. Numerous studies have reported that the source of antimicrobial peptides is the epidermal keratinocyte [48,49]. LL-37 antibacterial peptide was shown to be inducible [49], while antileukoprotease is constitutively produced in human keratinocytes. Human beta defensin-2 and 3 are inducible,

transcriptionally regulated antimicrobial peptides expressed by keratinocytes.

It is possible that the invading pathogens in the epidermis can induce the production of antimicrobial factors in keratinocytes such as NO, LL-37 and defensins through the keratinocyte mannose receptor. The role of KMR in the epidermal host defense is indirectly suggested by the fact that the place of production of keratinocyte-derived antimicrobial factors is the suprabasal layer of the epidermis, the same where the KMR is expressed [48,49]. The finding that epidermal keratinocytes express KMR also demonstrates that the epidermis is not only a passive physical barrier but functions as an active immune organ. In this organ suprabasal keratinocytes are able to kill invading microbes by producing antimicrobial factors and thereby prevent infections.

6. SUMMARY

1. We have developed a model using the immortalized HaCaT keratinocytes, for investigating markers that are associated with differentiation and proliferation of keratinocytes.

2. We have obtained data suggesting that α5 integrin, keratin 1 and keratin 10 proteins are not only markers of proliferation and differentiation of HaCaT keratinocytes, but important regulators of these processes.

3. We have shown that serum factors and cell density regulate the process of keratinocyte proliferation and differentiation (possibly besides other pathways) through the regulation of the mRNA and protein expression of these genes.

4. Our results suggest that cyclin D1 and cyclin D2 have different functions in human keratinocytes. Cyclin D1 expression is specific for G0/G1/S transition when quiescent keratinocytes enter the cell cycle, while cyclin D2 regulates the G1/S transition of already proliferating keratinocytes.

5. We have demonstrated the presence of a mannose receptor on the surface of keratinocytes and started its molecular characterization. The keratinocyte mannose receptor has a molecular weight of ~200 kD and indirect evidences suggest that it is homologous but not identical to the macrophage mannose receptor.

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